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13. ABSTRACT *(Maximum 200 Words)*

The hypothesis of my project is the behavior of an invasive tumor cell is largely determined by a collection of proteins on the surface of metastasis cell. I proposed to employ phage display single-chain variable fragment antibody library, mass spectrometry and antibody chip methodologies to study the molecular mechanisms of metastasis in breast carcinoma MDA-MB-435 cell line. During the first year of this grant, I have constructed a phage display scFv antibody library to the plasma membrane proteins of this cell line. I panned the library on intact cells to enrich population of phage-scFvs that are composed of antibodies to cell surface proteins of MDA-MB-435 cell. 5.6-fold, 14-fold and 20-fold enrichment was obtained as compared with background binding when three rounds subsequent pannings were performed. The specificity of 705 individual phage clones was tested by an ELISA assay. Forty-six of seven hundred and five phage clones showed 2.5-fold greater specificity to MDA-MB-435 cells as compared with normal mammary epithelial cells. Ten of these clones bind to MDA-MB-435 tumor cells in flow cytometry assays. These clones are being sub-cloned in preparation for expression as soluble scFv antibodies. The recombinant scFvs will be used to identify the target antigens.

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INTRODUCTION

Since breast cancer is a common form of cancer among women, and metastasis of the cancer to distant sites is the major cause of mortality in these women, it is vitally important to understand the mechanisms involved in a tumor's progression to the metastasis phenotype.

Due to the multifactorial and complex nature of tumor metastasis process, I proposed to develop a novel methodology for studying the tumor cell as a biological system. I am focusing on the proteins at the plasma membrane of the invasive and non-invasive tumor cell because these proteins are responsible for many of the phenotypic changes that occur during the transformation of the tumor cell to one with metastatic potential. The disciplines of phage display, mass spectrometry and micro-array technologies are combined in my proposal to develop a methodology for quantifying thousands of plasma membrane proteins simultaneously and identifying a collection of target antigens. By comparing of the plasma membrane protein profiles of phenotypically distinct cell lines(invasive breast cancer cell line, non-invasive breast cancer cell line and normal mammary epithelial cell line), I will decipher the subset of proteins that are required for a tumor to progress to the metastatic phenotype.

BODY

During the period of July 1,2001- June 30,2002, my efforts concentrated on aims one and two of my original proposal.

Aim One:
Select for profiles of scFv antibodies that bind to antigens on the surface of invasive MDA-MB-435 cells and non-invasive MCF-7 breast carcinoma cell lines.

I have constructed phage display scFv antibody libraries targeted to the plasma membrane proteins of invasive MDA-MB-435 and non-invasive MCF-7 breast carcinoma cell lines. In order to enrich for phage-scFvs that bind to the invasive breast carcinoma cells (MDA-MB-435), I performed three rounds of phage panning on intact MDA-MB-435 cells.

Briefly, $7x10^{11}$ purified phage were incubated for 2 hours with $1x10^{6}$ MDA-MB-435 cells at 4°C. After thorough washing of the cell supernatant, the bound phage were eluted by incubation of the cells for 10 minutes in 200ul of pH 2.2 elution buffer at room temperature. After neutralization of the elution solution with 1M Tris pH 8.0, the eluted phage were used to infect exponentially growing XL1-Blue cells. An aliquot was diluted and plated on the antibiotic agar for determining output phage titer, and the rest of the phage were rescued using helper phage (VCS-M13) and then amplified to produce the "enriched" phage-scFvs library. $7x10^{11}$ purified phage from the enriched library were used for second and third round of panning following the same procedure.

The three rounds of panning led to a 20-fold enrichment of phages that bind to the cell surface proteins of MDA-MB-435 cells in the third round of panning. Therefore, I have already succeeded in selecting a profile of scFv antibodies to the cell surface proteins of MDA-MB-435 cells. With the same procedure I also generated a panel of phage display scFv antibodies to the cell surface proteins of non-invasive breast carcinoma MCF-7 cells.

Aim Two:
Obtain individual scFv antibody clones that recognize proteins on the surface of invasive and non-invasive breast carcinoma cells.

The phage-cell ELISA assay was applied firstly to select individual phage clones displaying antibodies against cell surface proteins on the breast carcinoma cells. I picked up individual phage-infected bacteria colonies on the titration plates from third round panning, then inoculated into individual well of flat bottom 96-well plate and amplified phage overnight in 37°C incubator. After overnight incubation, I spun down bacteria and transferred 15ul phage supernatant into a v-bottom 96-well plate containing $2x10^4$ MDA-MB-435 cells per well in 100ul binding buffer, or the same amount of human normal mammary epithelial cells (HMEC) in 100ul binding buffer, to be used as a negative control. I incubated the cells and phages at 4°C for two hours with gentle shaking. After thorough washing of the cell pellet, the mouse anti-M13-antibody and HRP- conjugated goat anti-mouse antibody were used as primary antibody and secondary antibody separately to detect the binding of the phages to cells. The signal was measured in a microtiter plate reader at 490 wavelength. Forty-six out of seven hundred and five clones displayed 2.5-fold greater signal on MDA-MB-435 cells than on HMEC cells were selected for further investigation.

The selected clones were further characterized by flow cytometry. Forty-six clones were tested for their ability to bind to MDA-MB-435 cells. Briefly, $1x10^{12}$ purified phage were incubated with $1x10^6$ cells for two hours on ice. Following thorough washing, mouse anti-M13 antibody and FITC labeled goat anti-mouse IgG were used as primary and secondary antibody to detect the phage bounder. 10 clones showed specific binding to MDA-MB-435 cells, but not to HMEC cells. Flow cytometry data on one of those 10 clones is shown in figure 1.

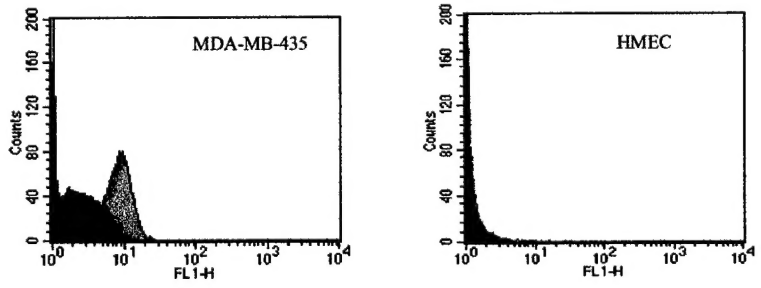

Figure1: Binding of #393 phage clone to MDA-MB-435 cells and HMEC cells

The result shows that the mean fluorescence intensity for MDA-MB-435 cells is 2.22-fold greater than that of the negative control; the mean fluorescence intensity for human normal mammary epithelial cells is 1.06-fold greater than that of the negative control. Therefore, these scFvs exhibit some degree of binding to the plasma membrane proteins of MDA-MB-435 cells.

To characterize the diversity of the panel of selected scFv clones, I sequenced the above mentioned 10 clones and aligned scFv sequence using Megalign software. Eight out of ten clones showed unique scFv sequences, showing that they are different antibodies.

To identify the target proteins of each antibody, I expressed them as recombinant fusion proteins with a His-Tag. Primers were designed and PCR was applied to amplify corresponding scFv. Then I sub-cloned the fragment into PET-20b(+) vector and transformed the cloning construct into Origami(DE3) cells for expressing soluble histidine-tagged scFv. Small-scale expression was performed to allow me to visualize 30KD fused histidine-scFv protein by Western Blot probing with anti-histidine antibody. Afterwards, I expressed His-scFv protein in large-scale, and purified fused protein using ProBond purification system. ProBond resin has a high affinity for the six tandem histidine residues. The fused His-scFv protein was eluted from ProBond resin by washing the resin with pH3.0 buffer. Following dialysis and concentration, I performed Coomassie staining and Western Blot to evaluate the yield, purity and property of the purified His-scFv protein. Each scFv was expressed and purified in milligram quantities and at high purity.

I am currently performing immunoprecipitation studies, in which I incubated His-scFv fused protein and breast carcinoma cell lysate overnight at 4°C, then incubated complex of His-scFv and corresponding bound antigen with ProBond resin. Following thorough washing, the precipitated antigen was eluted with pH3.0 buffer and loaded onto an 8% SDS-PAGE gel. After silver staining the gel, the protein band of interest will be excised and identified by mass spectrometry. The identification of a collection of plasma membrane proteins of invasive cancer cell line will contribute to better understanding the molecular mechanisms of metastasis.

Key Research Accomplishments:

- I have constructed phage display scFv antibody libraries targeted to the plasma membrane proteins of invasive MDA-MB-435 and non-invasive MCF-7 breast carcinoma cell lines.

- I have succeeded in selecting a profile of phages displaying scFv antibodies to the cell surface proteins of MDA-MB-435 cells and MCF-7 by repeatedly panning phage library on intact cells.

- I developed the phage-cell ELISA assay. Seven hundred and five individual phage clones were tested for their binding ability to MDA-MB-435 cells with this ELISA assay. Forty-six of those clones showed specificity to the cell surface proteins on MDA-MB-435 cells.

- The specificity of ten of forty-six clones was confirmed by flow cytometry assay. The sequence analysis of those 10 clones showed that 8 clones have unique scFv antibody sequences.

- I expressed soluble scFv antibodies as recombinant fusion proteins with a His-Tag, so I can immunoprecipitate and identify the target protein of each antibody.

CONCLUSIONS

The objectives of my study were:

- To develop an antibody chip for evaluating thousands of plasma membrane proteins (known or unknown) simultaneously.
- To understand the molecular mechanisms of metastasis by comparing of a collection of plasma membrane proteins of phenotypically distinct cell lines.

During the first year of my fellowship, I have successfully constructed phage display single-chain variable fragments(scFv) antibody libraries to the plasma membrane proteins of invasive and non-invasive breast cancer cell lines. As expected, I enriched the population of phage-scFv that are composed of antibodies targeting cell surface proteins on invasive breast cancer cell line (MDA-MB-435 cell) after I panned the original library on intact MDA-MB-435 cell. The binding specificity of individual phage clone from enriched library was characterized using phage-cell ELISA and flow cytometry assay. Therefore, I believe that I have already succeeded in selecting a profile of scFv antibodies to the cell surface proteins of MDA-MB-435 cells. The result also demonstrates it is possible to develop an antibody chip to study thousands of plasma membrane proteins (known or unknown) of phenotypically distinct cell lines by combining phage display and micro-array technologies .

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